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Antibacterial activity of endophytic fungi isolated from mangroves of Jaffna Peninsula, Sri Lanka

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Mangroves are plant communities growing in the intertidal zone of tropical to subtropical coastal rivers. Some endophytic fungi which live in the tissues of mangrove plants produce some biologically active substances. By screening these biologically active substances some researchers have found that these substances have antimicrobial activity. This research is aimed to determine the antibacterial activity of endophytic fungi isolated from leaves of mangrove plants *Excoecaria agallocha, Avicennia marina, Rhizophora mucronata* and *Lumnitzera racemosa* in Sarasalai area in Jaffna Peninsula in Sri Lanka. Various species of endophytic fungi were isolated from the leaves of mangrove plants and identified based on morphological characteristics. Five fungal species were isolated from *E. agallocha* four from *R. mucronata, A. marina* and two from *L. racemosa.* Fifteen endophytic fungi were tested against six selected bacteria for their antagonistic effect. Antibacterial activity was tested against *Escherichia coli, Bacillus* sp*, Klebsiella* sp*, Pseudomonas* sp*, Staphylococcus* sp. and *Proteus* sp. using disc diffusion assay. Almost all endophytic fungi inhibited the growth of bacteria. *Aspergillus flavus* had the highest amount of inhibition against *E. coli*, *Pseudomonas* and *Staphylococcus* sp. *Aspergillus tamari* had higher amount of inhibition against *Klebsiella* sp. Few other species of *Aspergillus* also showed higher inhibitory activity against different bacteria when compared to other endophytic fungi.

Key words: Mangrove, endophytic fungi, bacteria.

INTRODUCTION

Mangrove plants grow well in between sea and terrestrial ecosystem that contain brackish water. Mangroves live in wide range of salinities, tidal amplitudes, changes in sea level, winds, high temperatures, muddy and anaerobic soil conditions. They are well adapted for their extreme environmental conditions. In addition, most mangrove species are used as medicinal plants and also they have antimicrobial properties. These mangroves contain bioactive compounds that have potential antimicrobial, antiviral, anticancer, antidiabetic, antimalarial and antioxidant compounds (Zhang et al., 2009). Previous studies showed that most of the bioactive compounds were derived from the interaction between plants and microbes such as bacteria and endophytic fungi (Rossiana et al., 2016). Endophytic microorganisms grow within tissues of higher plants as facultative saprophytic, parasitic, mutualistic and commensalistic symbioses. These microorganisms grow intracellularly or intercelullarly in the tissues of higher plants without causing any symptoms on the host plants in which they live (Molina et al., 2012). Endophytic microorganisms are generally capable of producing bioactive compounds

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similar to their host plants (Nurhajati, 2011). Several studies have found that the endophytic fungi are one of the main sources of producing new antibiotics (Zhang et al., 2009). Endophytic fungi have been widely investigated as source of bioactive compounds (Bills et al., 1991). Most of these bioactive compounds have antimicrobial activity. The objective of this study was to isolate and identify endophytic fungi from selected mangroves and to test the biological activity of fungal isolates.

METHODOLOGY

The mangroves which are common in Sarasalai area in the Northern part of Sri Lanka were selected for this study. Leaves were collected from mangroves namely *Excoecaria agallocha, Avicennia marina, Rhizophora mucronata* and *Lumnitzera racemosa* at five random sites in the area during July before the rainy season. Three plants were selected from each mangrove species. The identification was based on the herbarium specimens (M 23/1900) available in the Department of Botany University of Jaffna Sri Lanka and assistance from a taxonomist. The mangrove specimens collected for this study were preserved as herbarium and maintained in the laboratory for future reference.

Mature leaves (3-4) from the selected mangrove plants were collected into sterile polythene bags in the field. After reaching the laboratory, the leaves were immersed in Sodium hypochloride for 1-2 min for sterilization. The leaves were washed with sterile water 3 times. Four small segments (about 4 mm x 4 mm size) from each leaf in between the mid rib and periphery were cut using a sterile razor. Potato Dextrose Agar (PDA 39 g/L) medium was prepared in Petri dishes with the addition of streptomycin to prevent the growth of bacteria. Leaf segments were placed on the surface of PDA. The plates were incubated at room temperature for 2-3 days to observe fungal growth. The individual fungi were sub cultured until pure fungal isolates were obtained. Microscopic morphology and macroscopic characters were used for the examination of fungal cultures in the laboratory. Fungi were identified using standard keys available for fungi (Pitt and Hocking, 1997).

Discs (5 mm diameter) of 5 days old fungal culture were inoculated into 250-mL conical flasks containing 50 mL of Potato Dextrose Broth medium (PDB). The conical flasks were placed on a thermostatic shaker at 180 rpm at 28°C for 7 days for fermentation.

The cultured fungal mats were filtered through cheese cloth. The filtrate (50 mL) was transferred into separating flask. The crude metabolites were extracted using ethyl acetate at room temperature. The extracted ethyl acetate fractions were pooled into a conical flask, dried over anhydrous $MgSO_4$ and evaporated by using rotary evaporator. The crude extract was dissolved in Dimethyl Sulphoxide (DMSO).

The bacterial cultures used in this study were obtained from the bacterial culture collection available in the laboratory in the Department of Botany University of Jaffna. The choice of bacteria was based on the previous studies made on the antibacterial activity. Bacteria (*Escherichia coli, Bacillus* sp*, Klebsiella* sp*, Pseudomonas* sp*, Staphylococcus* sp and *Proteus* sp) were streaked on the surface of the Nutrient Agar (NA) (28 g/L) medium. The plates were incubated at 37°C for overnight. After the incubation, a loop full of young bacterial culture (16-24 h old) from the isolated colony was transferred into the universal bottle containing sterile distilled water. It was stirred well by using vortex stirrer. Suspension was prepared which contain 10^5 CFU/ml from each bacterial culture.

Nutrient agar (NA) medium was prepared. 0.1 ml of bacterial suspension was transferred into the centre of the NA plates separately by using sterile pipette. Thereafter, the suspension was spread all over the surface of the NA medium by using sterile glass spreader.

A 50 µL of fungal metabolite extract was added into a sterile paper disc (5 mm diameter, Whatman No. 1). The paper disc was placed on NA plates which were surface inoculated with bacterial cultures. The antibacterial agent ampicillin (60 µg/mL) was used as a positive control and DMSO was used as a negative control. The plates were incubated at 37°C for 24-48 h and inhibition zones were measured. The experiment was carried out in triplicates.

Fifteen endophytic fungi were tested against six selected bacteria for their antagonistic effect. Positive and negative controls were also maintained. The obtained results were analyzed by 2-way ANOVA.

RESULTS AND DISCUSSION

A total of 15 different species of endophytic fungi were isolated from the leaves of mangrove plants. Five different fungal species were isolated from *Excoecaria* sp., four from *Rhizophora* sp and *Avicennia* sp and two from *Lumnitzera* sp (Figures 1 and 2). The features of identification are given in Table 1. The mean values of the inhibition zones are given in the Table 2.

Almost all fungi inhibited the growth of *E. coli*. Endophytic fungus *Aspergillus flavus* (F1) has the higher amount of inhibition against *E. coli*. Endophytic fungus *Aspergillus* sp (F5) has the lowest amount of inhibition against *E. coli*. Most of the fungus inhibited the growth of *Bacillus* sp. Endophytic fungus *Aspergillus* sp (F3) has the higher amount of inhibition against *Bacillus* sp. Endophytic fungus *Aspergillus* sp (F5) and *Epicoccum nigrum* (F14) had the lowest amount of inhibition against *Bacillus* sp. Almost all fungus inhibited the growth of *Klebsiella* sp. Endophytic fungus *Aspergillus tamarii* (F2) has the higher amount of inhibition against *Klebsiella* sp. Endophytic fungus *Chaetomium* sp (F7) has the lowest amount of inhibition against *Klebsiella* sp. Almost all fungus inhibited the growth of *Proteus* sp. Endophytic fungus *Aspergillus* sp (F4) has the higher amount of inhibition against *Proteus* sp. Endophytic fungus *Mucor* sp (F6) has the lowest amount of inhibition against *Proteus* sp. Almost all fungus inhibited the growth of *Pseudomoas* sp. Endophytic fungus *A. flavus* (F1) has the higher amount of inhibition against *Pseudomoas* sp. Endophytic fungus *E. nigrum* (F15) has the lowest amount of inhibition against *Pseudomoas* sp. Almost all fungus inhibited the growth of *Staphylococcus* sp. Endophytic fungus *A. flavus* (F1) has the higher amount of inhibition against *Staphylococcus* sp. Endophytic fungus *E. nigrum* (F14) has the lowest amount of inhibition against *Staphylococcus* sp.

At P=0.05, statistical analysis showed that, there is no interaction between mean values of diameter of fungus. Also, there is no significant difference (P=0.05) in the diameters of clear zones of different fungi tested in this study. Highest antagonistic activity was shown by *Aspergillus* sp. (F3) against *Pseudomonas* sp. whereas

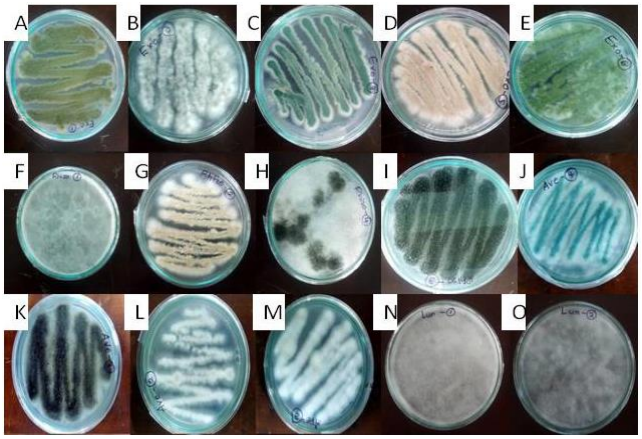

Figure 1. (A-E) Fungus isolated from *Excoecaria* in PDA medium A- *Aspergillus flavus ;* B- *Aspergillus tamari ;* C- *Aspergillus* sp. 1; D- *Aspergillus* sp. 2; E- *Aspergillus* sp. 3; (F-I) Fungus isolated from *Rhizophora* F- Unknown G- *Aspergillus* sp. 4; H- *Aspergillus niger* I- *Mucor* sp 1(J-M); Fungus isolated from *Avicennia* J- *Aspergillus* sp 5; K- *Mucor* sp 2; L- *Chaetomium* sp M- *Aspergillus* sp 6 (N-O) Fungus isolated from *Lumnitzera* N- *Epicoccum nigrum* O- *Epicoccum nigrum.*

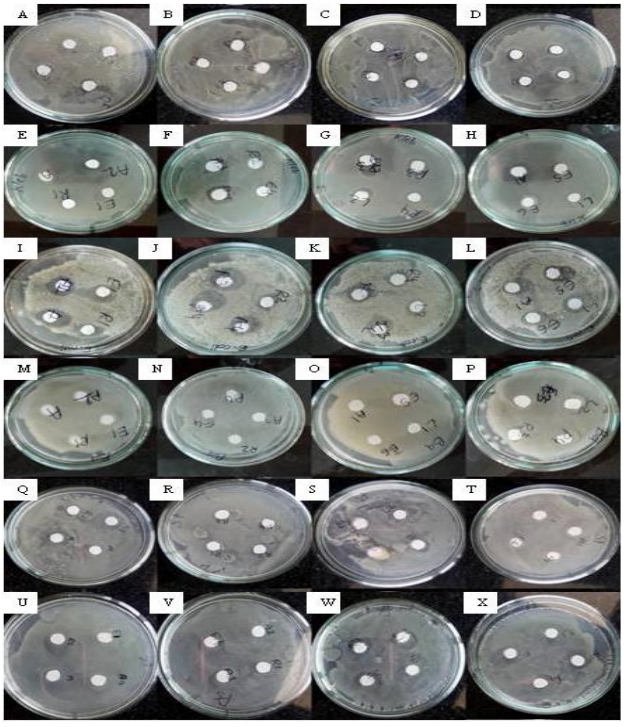

Figure 2. Antibacterial activity of endophytic fungi. (A-D) Inhibition zones of all fungi on NA plate surface inoculated by *Proteus* sp.; (E-H) Inhibition zones of all fungi on NA plate surface inoculated by *Klebsiella* sp.; (I-L) Inhibition zones of all fungi on NA plate surface inoculated by *Escherichia coli.;* (M-P) Inhibition zones of all fungi on NA plate surface inoculated by *Bacillus* sp.; (Q-T) Inhibition zones of all fungi on NA plate surface inoculated by *Staphylococcus* sp.; (U-X) Inhibition zones of all fungi on NA plate surface inoculated by *Pseudomonas* sp.

Table 1. The features of Identification of endophytic fungi.

Fungus	Colony character	Microscopic character	Name of the fungus
A	Colony Lime green in colour. Cottony, powdery appearance. Edge is white colour. Middle of the colony is raised. Backside of the colony is white in colour.	Conidia are round shape. Loosely radiate phialiodes on most of the vesicle. Hyphae are septate and branched	*Aspergillus flavus*
B	Colony is green in colour. No mycelium. Powdery appearance and irregular form. Middle of the colony is raised. Backside of the colony contains black colour spots.	Hyphae are septate and branched	*Aspergillus tamari*
C	Colony is bluish green in colour. Edge of the colony is white in colour. Yellow colour spots present. Backside of the colony is white colour and black colour spots present.	Conidia are round shape. Hyphae are aseptate and unbranched	*Aspergillus* sp. 1
D	Light brown colour. Margin of the colony is white colour. Backside of the colony is yellow colour	Hyphae are septate and branched	*Aspergillus* sp. 2
E	Colony is green in colour.Backside of the colony is white colour. Middle raised.	Hyphae are septate and branched	*Aspergillus* sp. 3
F	Whitish colour colonies present. Later it changes in to blackish colour. Backside of the colony is black colour. Black colour spores are present.	Hyphae are aseptate and unbranched	Unknown
G	Light brown colour. Margin of the colony is white colour. Backside of the colony is yellow colour	Hyphae are septate and branched	*Aspergillus* sp. 4
H	White colour mycelium with black colour spores. Backside of the colony is white colour.	Hyphae is branched and septate.conidia are globose shape	*Aspergillus niger*
I	Colonies are black colour. Margin is white colour. Backside of the colony is white colour.	Aseptate broad hyphae, sporangiophore round slightly elongated	*Mucor* sp.
J	Colonies are bluish green in colour. Margin is white colour. Edges of the colonies are smooth. Backside of the colony is white colour	Hyphae are septate and branched.	*Aspergillus* sp. 5
K	Colonies are black colour. Margin is white colour. Backside of the colony is white colour.	Aseptate broad hyphae, sporangiophore round slightly elongated	*Mucor* sp.
L	Colonies are white in colour. Middle raised. Filamentous mycelia present. Backside of the colony is white colour.	Hyphae are septate and branched	*Chaetomium* sp.
M	Colonies are milky white colour. Middle raised. Backside of the colony is white colour.	Hyphae are septate and branched	*Aspergillus* sp. 6
N	White colour mycelium present. Cottany appearance. Backside of the colony is black colour.	Conidia are black colour warted and spherical Hyphae of the mycelium are septate and branched.	*Epicoccum nigrum*
O	White colour mycelium present. Cottany appearance. Backside of the colony is black colour.	Conidia are black colour warted and spherical. Hyphae of the mycelium are septate and branched.	*Epicoccum nigrum*

lowest antagonistic activity was shown by *E. nigrum* (F14) against *Bacillus* sp. The interaction between fungi and bacteria is shown in Figures 1 and 2.

Conclusion

Different mangroves used in this research study had different endophytic fungi or different strains of the same endophytic fungus. This study was done during the dry period or just before the rainy season. Future studies should be carried out to correlate the seasonal variations and the presence

Table 2. Mean values of the inhibition zones of endophytic fungi (mm).

Fungi	*E. coli*	*Bacillus*	*Klebsiella*	*Pseudomonas*	*Proteus*	*Staphylococcus*
F1	2.0333	0.5250	1.1583	2.5833	1.2583	1.6667
F2	1.8083	0.9750	1.8083	2.0417	1.1833	0.7917
F3	1.5750	1.3250	1.5417	2.7250	1.1583	1.2083
F4	1.7250	0.9583	1.5917	1.7250	1.4750	1.4667
F5	0.5583	0.0000	1.4583	1.2917	1.2917	0.9167
F6	1.7167	0.5083	1.5583	2.0417	1.0667	1.3167
F7	1.9250	0.5167	0.9167	2.3917	1.1917	1.1104
F8	1.7833	0.4500	1.5833	2.4750	1.2667	0.6917
F9	1.7500	0.9250	1.6833	2.5333	1.2333	1.3750
F10	1.9833	0.5917	1.5583	1.9083	1.2333	1.4917
F11	1.4750	0.8917	1.4750	2.1083	1.2083	1.2500

of endophytic fungi. It can be concluded that endophytes are rich sources of bioactive natural products with promising applications in development of pharmaceutical and industrial compounds. The fungal metabolites in the crude form have been used in this study. Further research should be carried out with the purified extracts. The follow up study would be the identification of the endophytic fungi at the molecular level to confirm the species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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